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Underground cryptic speciation within the Maghreb: Multilocus phylogeography sheds light on the diversification of the checkerboard worm lizard *Trogonophis wiegmanni*

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ABSTRACT

Biogeographic and evolutionary patterns in the North African portion of the Western Palaearctic are poorly known. A high fraction of undescribed diversity is expected in this region, especially in groups such as reptiles. Here we used mitochondrial (*12S*, *16S*, *cytb*) and nuclear (*pomc*, *rag2*, *cmos*) markers and morphological data to investigate phyletic diversification and phylogeographical structure in the amphisbaenian *Trogonophis wiegmanni* endemic to the Maghreb. Phylogenetic and molecular dating analyses based on gene trees and species trees support three deeply divergent lineages of Pliocene origin, two in Morocco and one in central Algeria and Tunisia. Parapatry, reciprocal monophyly, high genetic divergence and limited morphological differentiation between them suggest that these lineages represent independent cryptic taxonomic units. Emerging lines of evidence from this study and from available literature on Maghreb taxa support (i) a major biogeographic break between western and eastern Maghreb and (ii) a role of the Atlas as a biogeographic divide within the western Maghreb (Morocco). The origin of these biogeographic units is probably associated with the evolutionary events prompted by the Late Miocene palaeogeographic setting and later by Plio-Pleistocene climatic changes and their interplay with prominent orographic barriers within North Africa.

1. Introduction

Knowledge on organism diversification and biodiversity patterns is far from complete even for some of the best-studied regions such as the Western Palaearctic (Ficetola et al., 2013). Here, while taxa of the European side of the Mediterranean Basin have been the subject of intensive phylogeographic surveys, comparatively little is known regarding the North African counterpart, the Maghreb (Hewitt, 2004; Schmitt, 2007; Habel et al., 2009; Husemann et al., 2014). In particular, the extent of reptile diversification within this region is highly underestimated as a result of limited surveys due to the remoteness of some regions, the roughness of the landscape and the lasting political instability (Ficetola et al., 2013; see also Harris and Froufe, 2005). Furthermore, most studies in the region revealed the occurrence of cryptic species, which can only be detected by molecular tools (e.g. Busack, 1988; Perera and Harris, 2010a; Barata et al., 2012).

Strong phylogeographic structure and cryptic diversity are commonly observed in organisms with low dispersal ability and a habitat-specialised morphology such as fossorial reptiles. In these organisms, speciation and genetic divergence are typically uncoupled from morphological diversification (e.g. Kearney and Stuart, 2004). Thus, while the effect of long-term historical isolation between populations may be detected in their gene genealogies in the form of allopatric phylogenetic clades, morphological features specialised to the fossorial lifestyle are maintained over time (Gans, 1977). Indeed, recent molecular studies on amphisbaenian populations from the Western Palaearctic have revealed multiple evolutionary lineages separated by prominent biogeographical barriers within widespread morpho-species (Busack, 1988; Mendonça and Harris, 2007; Albert and Fernández, 2009; Sindaco et al., 2014; Sampaio et al., 2015).

Amphisbaenians (worm lizards) are strict subterranean reptiles. Phenotypic adaptations to burrowing lifestyle include elongated cylindrical bodies, rudimentary eyes, and reduced or absent limbs (Gans, 1977). In the Western Palaearctic worm lizards are represented by the

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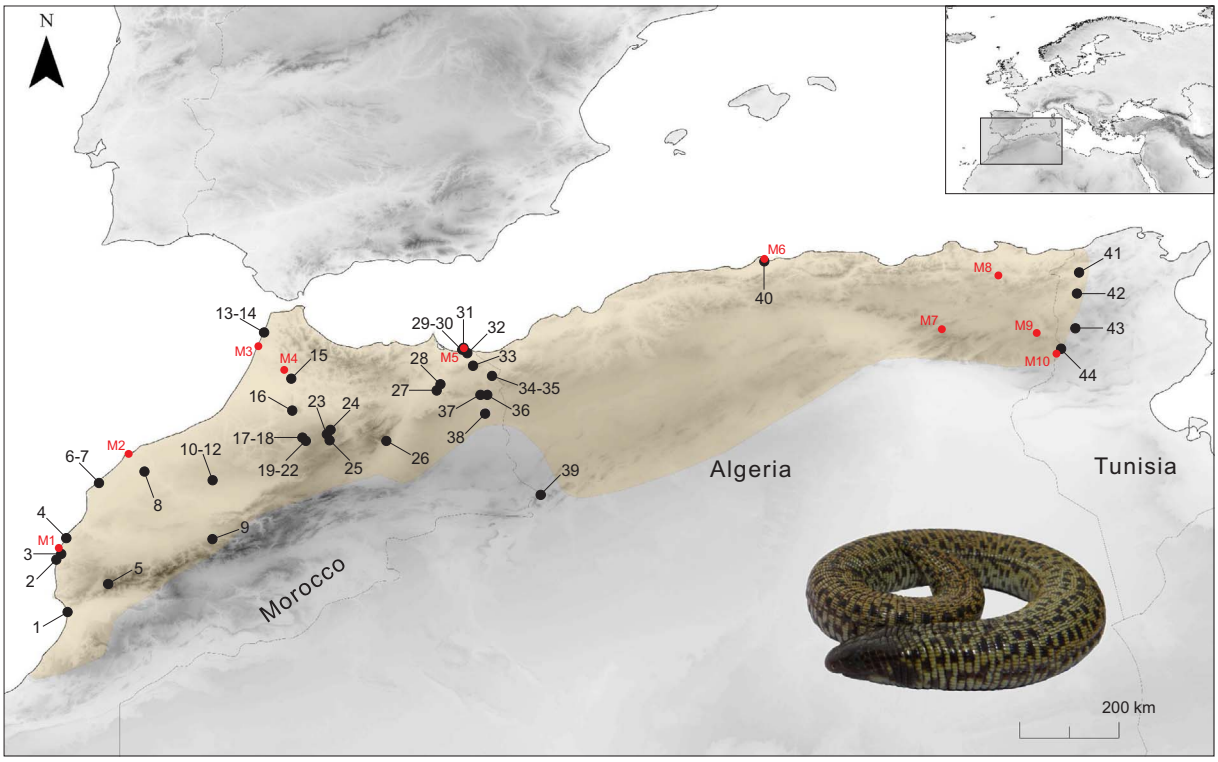

Fig. 1. Maps of the study area with the geographic location of the *Trogonophis wiegmanni* samples analysed. The brown-shadowed area in the map represents the species distribution range according to IUCN (2009); the box in the upper-right part of the map shows the study area in the context of the Western Palaearctic. Black dots indicate samples used for genetic analyses (1–44); red dots indicate samples used for morphological analyses (M1-M10; the Tunisian sample M11 is not represented as the precise locality is not known). Superimposed in the bottom-right part of the map is represented an individual of *T. w. wiegmanni* (photo by D. Salvi). Additional information on samples are reported in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

families Blanidae, with seven species in the circum-Mediterranean genus *Blanus,* and Trogonophidae, with six species in four genera distributed in western Iran and the Arabian Peninsula, the Horn of Africa and North Africa (Uetz et al., 2017). In this study, we focused on the phyletic diversification and phylogeographical structure of the checkerboard worm lizard *Trogonophis wiegmanni*, so called for its distinctive checkered pattern of dark and light scales. This species is the only representative of this genus and the only representative of the family Trogonophidae in North Africa, being distributed from southwest Morocco to northeast Tunisia (Fig. 1). It is found in a variety of habitats with sandy soils and rocks in areas with Mediterranean and sub-arid bioclimatic regimes (Schleich et al., 1996; Sánchez and Escoriza, 2014). Two subspecies have been described, only distinguished by colouration: *T. w. elegans*, also called 'mauve form' (with a white to pinkish ground colour) endemic to the central and western Morocco region north and west of the Atlas Mountains; and *T. w. wiegmanni*, the 'yellow form' (with a yellowish ground colour) ranging from the eastern slope of the Rif and Middle Atlas Mountains of Morocco to western Tunisia (Bons and Geniez, 1996; Schleich et al., 1996). However, exceptions to this simple geographic pattern of phenotypic variation have been reported (Boulenger, 1891; Doumergue, 1901). A preliminary mitochondrial assessment showed high genetic divergence between *T. w. elegans* and *T. w. wiegmanni* from Morocco and between them and a single individual sampled in Tunisia (Mendonça and Harris, 2007), suggesting the existence of undescribed diversity within this group.

Here we use dated phylogenies based on DNA sequence data from multiple mitochondrial and nuclear markers and morphological analyses to assess (i) whether previously identified mitochondrial lineages within *T. wiegmanni* correspond to distinct species and to what extent they are consistent with the described subspecies, and (ii) to discuss in a comparative framework the temporal and spatial pattern of the main cladogenetic events in *Trogonophis* and other North African taxa and their association to past environmental changes in North Africa. The main aim of this study is to identify independent evolutionary lineages or cryptic taxonomic units within *Trogonophis wiegmanni* and to contribute to our understanding of the biogeographic and biodiversity patterns within the Maghreb.

2. Material and methods

2.1. Genetic data collection

We sampled a total of 44 *Trogonophis wiegmanni* specimens from 35 localities across the Maghreb (Table 1; Fig. 1). Individuals were assigned to either subspecies based on colouration. Sampling was fairly complete in the Tunisian and Moroccan range, with a denser sampling in the putative contact zone between the two subspecies, but very limited for Algeria due to security concerns. *Diplometopon zarudnyi* (family Trogonophidae) was used as an outgroup in the phylogenetic analyses (Vidal et al., 2008; Zheng and Wiens, 2016).

Genomic DNA was extracted from alcohol-preserved tail-tip muscle using the DNeasy Blood & Tissue kit (Qiagen®, Valencia, California) following the manufacturer's protocol. We amplified and sequenced three mitochondrial gene fragments – 12S rRNA (*12S*), 16S rRNA (*16S*) and cytochrome *b* (*cytb*) – and three nuclear protein-coding genes – propiomelanocortin (*pomc*), recombination activation gene 2 (*rag2*) and oocyte maturation factor (*cmos*). We selected these gene fragments to allow comparisons with a previous study on *T. wiegmanni* (*12S* and *16S*; Mendonça and Harris, 2007) and because they have been successfully employed in previous intraspecific and interspecific studies on squamates including amphisbaenids (e.g. Mott and Vieites, 2009; Vidal et al., 2008; Salvi et al., 2013, 2017; Sampaio et al., 2015). Amplifications were performed through standard Polymerase Chain Reaction (PCR) in final volumes of 25 μL, containing 5 μL 5× reaction buffer,

Table 1
Geographic, morphotype and genetic information on the *Trogonophis wiegmanni* individuals analysed in this study. Sample codes correspond to the map in Fig. 1. Individual morphotype was assigned in the field to the subspecies *T. w. elegans* ('mauve form') or *T. w. wiegmanni* ('yellow form') or intermediate between the two (*T. w. elegans/T. w. wiegmanni*) based on the pattern of colouration following Schleich et al. (1996). GenBank accession numbers for each individual and gene fragment are provided. GenBank accession numbers followed by "*" correspond to the sequences published by Mendonça and Harris (2007) and the relative individual codes of the original publication are provided in brackets in the Collection code column.

Sample code	Collection code	Morphotype	Locality	Latitude/Longitude	*12S*	*16S*	*cytb*	*pomc*	*rag2*	*cmos*
1	SPM001686	*T. w. elegans*	Agadir (Morocco)	30.40/−9.60	MG661073	–	MG661129	–	–	–
2	DB11936	*T. w. elegans*	Sidi Kaouki (Morocco)	31.34/−9.80	MG661074	MG661103	MG661130	MG661167	MG661195	MG661223
3	DB20108	*T. w. elegans*	Ghazoua (Morocco)	31.44/−9.72	MG661075	MG661104	MG661131	MG661168	MG661196	MG661224
4	DB5172	*T. w. elegans*	Akermoud (Morocco)	31.74/−9.62	MG661076	MG661105	MG661132	–	–	–
5	DB1322	*T. w. elegans*	Tanout (Morocco)	30.90/−8.86	MG661077	MG661106	MG661133	MG661169	MG661197	MG661225
6	SPM003091	*T. w. elegans*	Oued -Rharg (Morocco)	32.74/−9.03	MG661078	MG661107	MG661134	MG661170	–	MG661226
7	SPM003090	*T. w. elegans*	Oued -Rharg (Morocco)	32.74/−9.03	MG661079	MG661108	MG661135	MG661171	MG661198	MG661227
8	DB4859 (TR1r)	*T. w. elegans*	Al Jadida (Morocco)	32.95/−8.21	EF545726*	EF545727*	MG661136	MG661172	MG661199	MG661228
9	DB2346	*T. w. elegans*	Iminifri (Morocco)	31.72/−6.97	MG661080	MG661109	MG661137	MG661173	MG661200	MG661229
10	DB4855 (TR2)	*T. w. elegans*	Oulad Brahim (Morocco)	32.79/−6.96	EF545712*	EF545713*	MG661138	MG661174	MG661201	MG661230
11	DB4856 (TR3)	*T. w. elegans*	Oulad Brahim (Morocco)	32.79/−6.96	EF545714*	EF545715*	MG661139	–	MG661202	MG661231
12	DB4857 (TR4)	*T. w. elegans*	Khouribga (Morocco)	32.79/−6.96	EF545716*	EF545717*	MG661140	MG661175	MG661203	MG661232
13	DB4858 (TR5)	*T. w. elegans*	Assilah (Morocco)	35.47/−6.04	EF545718*	EF545719*	MG661141	MG661176	MG661204	MG661233
14	SPM002114	*T. w. elegans*	Asilah (Morocco)	35.47/−6.03	MG661081	–	MG661142	–	–	–
15	DB2331	*T. w. weigmanni*	Ain Defali (Morocco)	34.63/−5.54	MG661082	MG661110	MG661143	MG661177	MG661205	MG661234
16	Tr6 (TR6)	*T. w. elegans/T. w. weigmanni*	Moulay Idriss (Morocco)	34.05/−5.52	EF545720*	EF545721*	–	–	–	–
17	DB962	*T. w. elegans*	Paysage d'Ito (Morocco)	33.55/−5.33	MG661083	MG661111	MG661144	MG661178	MG661206	MG661235
18	DB963	*T. w. elegans/T. w. weigmanni*	Paysage d'Ito (Morocco)	33.55/−5.33	MG661084	MG661112	MG661145	MG661179	MG661207	MG661236
19	DB27597	*T. w. elegans/T. w. weigmanni*	Paysage d'Ito (Morocco)	33.50/−5.28	MG661085	MG661113	MG661146	–	–	MG661237
20	DB25294	*T. w. elegans/T. w. weigmanni*	Paysage d'Ito (Morocco)	33.50/−5.28	MG661086	MG661114	MG661147	MG661180	MG661208	MG661238
21	DB25275	*T. w. elegans/T. w. weigmanni*	Paysage d'Ito (Morocco)	33.50/−5.28	MG661087	MG661115	MG661148	MG661181	MG661209	MG661239
22	DB25381	*T. w. elegans/T. w. weigmanni*	Paysage d'Ito (Morocco)	33.50/−5.28	MG661088	MG661116	MG661149	MG661182	MG661210	MG661240
23	DB1508	*T. w. elegans/T. w. weigmanni*	Lac Afourgaa (Morocco)	33.63/−4.90	MG661089	MG661117	MG661150	MG661183	MG661211	MG661241
24	DB15412	*T. w. weigmanni*	Ifrane (Morocco)	33.52/−4.85	MG661090	MG661118	MG661151	MG661184	MG661212	MG661242
25	DB25382	*T. w. elegans/T. w. weigmanni*	Ait Khalifa (Morocco)	33.69/−4.83	MG661091	MG661119	MG661152	MG661185	MG661213	MG661243
26	Tr72 (TR72)	*T. w. weigmanni*	Tirnest (Morocco)	33.05/−3.82	EF545724*	EF545725*	–	–	–	–
27	SPM002131	*T. w. weigmanni*	Tawrirt (Morocco)	34.42/−2.90	–	–	MG661153	–	–	–
28	DB3292	*T. w. weigmanni*	Talwat (Morocco)	34.52/−2.84	MG661092	MG661120	MG661154	MG661186	MG661214	MG661244
29	MNCN44398	*T. w. weigmanni*	Chafarinas islands (Spain)	35.18/−2.43	MG661093	MG661121	MG661155	–	–	MG661245
30	MC44Tw	*T. w. weigmanni*	Cap de l'Eau (Morocco)	35.15/−2.43	MG661094	–	MG661156	–	–	–
31	MC37Tw1	*T. w. weigmanni*	Cap de l'Eau (Morocco)	35.15/−2.43	MG661095	MG661122	MG661157	MG661187	MG661215	MG661246
32	Tr139R (TR139r)	*T. w. weigmanni*	Cherarba (Morocco)	35.10/−2.35	EF545734*	EF545735*	–	–	–	–
33	SPM002120	*T. w. weigmanni*	Ouled Herrou (Morocco)	34.86/−2.24	MG661096	–	MG661158	–	–	–
34	Tr761 (TR761)	*T. w. weigmanni*	Berkane Oujda (Morocco)	34.68/−1.90	EF545732*	EF545733*	–	–	–	–
35	Tr768 (TR768)	*T. w. weigmanni*	Berkane Oujda (Morocco)	34.68/−1.90	EF545730*	EF545731*	–	–	–	–
36	DB14682	*T. w. weigmanni*	El Aouinet (Morocco)	34.34/−2.11	MG661097	MG661123	MG661159	MG661188	MG661216	MG661247
37	DB14623	*T. w. weigmanni*	Jerada (Morocco)	34.34/−1.99	MG661098	MG661124	MG661160	MG661189	MG661217	MG661248
38	Tr62 (TR62)	*T. w. weigmanni*	Ain Beni Mathar (Morocco)	34.00/−2.02	EF545722*	EF545723*	–	–	–	–
39	DB3135	*T. w. weigmanni*	Ich (Morocco)	32.52/−1.01	MG661099	MG661125	MG661161	MG661190	MG661218	MG661249
40	SPM002541	*T. w. weigmanni*	Algiers (Algeria)	36.75/3.04	MG661100	MG661126	MG661162	MG661191	MG661219	MG661250
41	DB4	*T. w. weigmanni*	Bulla Regia (Tunisia)	36.56/8.75	MG661101	MG661127	MG661163	MG661192	MG661220	MG661251
42	DB4860 (TR2r)	*T. w. weigmanni*	Le Kef (Tunisia)	36.18/8.71	EF545728*	EF545729*	MG661164	MG661193	MG661221	MG661252
43	DS-Tun	*T. w. weigmanni*	Talah (Tunisia)	35.55/8.68	MG661102	MG661128	MG661165	MG661194	MG661222	MG661253
44	T31-47 Trogwieg1	*T. w. weigmanni*	Bou Chebka (Tunisia)	35.17/8.42	–	–	MG661166	–	–	–

2–3 mM $MgCl_2$, 0.2–0.4 μM each dNTP, 0.2 μM each primer, 1 U of Taq polymerase and 0.5–1 μL DNA template. Primers and PCR conditions used for the amplification of the molecular markers are reported in Table S1.

2.2. Phylogenetic analyses

Multiple DNA sequences alignments were performed in Geneious v6.0 (www.geneious.com) using the Geneious Alignment algorithm. The number of variable positions and average genetic distance between sequences (uncorrected *p*-distance with pairwise-deletion option) were calculated in MEGA v6 (Tamura et al., 2013). Nuclear haplotype phase was inferred using the PHASE algorithm (Stephens et al., 2001) implemented in DNAsp v5 (Librado and Rozas, 2009). For each nuclear gene, the possible occurrence of recombination events was assessed using the Pairwise Homoplasy Index (phi) test (Bruen et al., 2006) implemented in SplitsTree4 v4.13.1 (Huson and Bryant, 2006).

We performed a Bayesian evolutionary analysis in BEAST v1.8.4 (Drummond et al., 2012) to estimate phylogenetic relationships among mitochondrial haplotypes and associate a time/age at each node of the phylogeny. We implemented the TrN + G model for the *12S*, the HKY + G+I for the *16S* and the HKY + G for the *cytb* partitions according to the best substitution models (BIC Criterion) selected by PartitionFinder v1.1.1 (Lanfear et al., 2012). We used a coalescent tree prior and a relaxed uncorrelated lognormal clock model. In order to calibrate the molecular clock and estimate the time to the most recent common ancestor (TMRCA) of the *T. wiegmanni* lineages, we used both available substitution rates for squamates and the time of the split between *T. wiegmanni* and *D. zarudnyi*. Vidal et al. (2008) estimated this split at 31.12 million years ago (Ma), with a credibility interval of 20.91–44.38 Ma, based on one fossil and two biogeographic calibrations within Amphisbaenidae. This time interval is consistent with (i.e. includes) the estimates for the same split obtained by later studies on squamate reptiles based on 9–14 fossil calibrations (Wiens et al., 2006; Mulcahy et al., 2012; Zheng and Wiens, 2016). Substitution rates for *12S* (0.00755; SD = 0.00247) and *cytb* (0.0228; SD = 0.00806) were estimated by Carranza and Arnold (2012) based on seven biogeographic calibrations (between lacertid, skink, and gecko taxa from the Canary Islands and the Mediterranean). In order to incorporate these priors and associated uncertainty, we defined a normal distribution on the root height with $\mu = 31.12$ and $\sigma = 6.04056$, and lognormal distributions on the *12S* and *cytb* substitution rates (parameter ucld.mean; *12S*: $\mu = 0.00755$, $\sigma = 0.15$; *cytb*: $\mu = 0.0228$, $\sigma = 0.175$). Independent preliminary analyses were run using either the root prior or the rate priors and then both combined in order to verify their compatibility and the consistency of results based on different calibration strategies. Each BEAST analysis was run twice, with 30 million iterations per run, sampling every 3000 steps. Convergence was assessed in Tracer v1.6 (available at http://beast.bio.ed.ac.uk/Tracer). Sampled trees after the burn-in (25%) from independent runs were combined in LogCombiner and used in TreeAnnotator to calculate Maximum Clade Credibility Trees and Bayesian Posterior Probabilities (BPP) of nodes. In addition, phylogenetic relationships between concatenated mitochondrial sequences were inferred using the Maximum Likelihood (ML) method implemented in RaxML v7.4.2 (Stamatakis, 2006) using the GTRGAMMA model for each of the three gene partitions, 100 random addition replicates and 1000 nonparametric bootstrap replicates.

Phylogenetic networks based on nuclear haplotypes were estimated using the statistical parsimony approach (Templeton et al., 1992) implemented in TCS (Clement et al., 2000), under the 95% probability criterion for a parsimonious connection. This method is particularly appropriate when few characters for phylogenetic analysis are available due to shallow levels of divergence (Posada and Crandall, 2001) as observed in the *pomc*, *rag2* and *cmos* datasets.

Finally, we estimated a multilocus species tree in ∗BEAST (Heled and Drummond, 2010) to infer relationships and divergence time of the main lineages recovered in single gene trees (see results). This allowed us (i) to combine the phylogenetic information of mitochondrial and nuclear datasets, (ii) to verify the consistency of mtDNA trees inferred using either a coalescent tree prior implemented in BEAST, or the multispecies coalescent prior implemented in ∗BEAST, (iii) to compare divergence time estimates based on gene trees or species tree approaches. For the ∗BEAST analyses we excluded samples from the areas of putative genetic admixture between lineages (samples 17–25 and 39, see results) as this approach assumes no gene flow between lineages (Heled and Drummond, 2010). We implemented the HKY model for *pomc* and *rag2* and the K80 model for the *cmos* dataset (selected by PartitionFinder) and a Yule tree prior; all remaining settings were identical to the BEAST analysis. ∗BEAST was run twice, with 100 million iterations sampled every 10,000 steps.

2.3. Morphological data collection

We examined 72 *Trogonophis* specimens deposited in the collections of the Muséum National d'Histoire Naturelle (Paris, France), the Natural History Museum (London, UK), the Museo Nacional de Ciencias Naturales (Madrid, Spain) and the Estación Biológica de Doñana (Seville, Spain). We included only specimens from localities that could be confidently assigned to each genetic lineage, i.e. nearby genotyped individuals (Figs. 1 and 2). Accordingly, 17 specimens (localities M6-M11) were assigned to the eastern *T. w. wiegmanni* lineage, 29 specimens (from locality M5, Chafarinas Islands) to the western *T. w. wiegmanni* lineage and 26 (from localities M1-M4) to the *T. w. elegans* lineage. We analysed five linear measurements and 11 meristic characters (7 scale counts and 4 categorical variables). A detailed list and definition of morphological variables is provided in Table 3. Lateral variables were always recorded from the right side of the specimen.

2.4. Morphological analysis

Quantitative variables (linear measurements and scale counts) were log transformed and tested for normality and homoscedasticity using Shapiro and Bartlett tests respectively. All continuous variables had similar variances among lineages (in all cases, $p > .05$), but several of them failed normality. For this reason, we used (non-parametric) permutational statistical approaches of analysis. Correlations between body size (SVL) and other continuous variables were assessed using Spearman tests. When correlations were significant, new size-corrected variables were computed through residuals correction (from linear regressions between the continuous variable and SVL). Differences among lineages and pairwise differences between lineages for each variable were assessed using permutational ANOVAs as implemented in the R package geomorph v3.0.2 (Adams and Otárola-Castillo, 2013). Regarding categorical variables, differences in frequencies between lineages were evaluated using Fisher exact tests with simulated *p*-values (based on 2000 replicates).

Multivariate analyses were performed to have a preliminary estimate of the phenotypic variation within *Trogonophis*. A Principal Components Analysis (PCA, prcomp function, R Core Team, 2015) was performed on continuous variables, namely four body measurements (SVL plus three size-corrected head measurements) and five scale counts (ULS and LLS were not included because they showed no variation). Categorical variables regarding scale position (CSLS, EXSLS, EXBPPF and EXPFB) were analysed via Multiple Correspondence Analysis (MCA) using the function MCA implemented in the R package FactoMineR (Lê et al., 2008).

All analyses were performed in R using the RStudio v0.99.903 interface (R Core Team, 2015; RStudio Team, 2015).

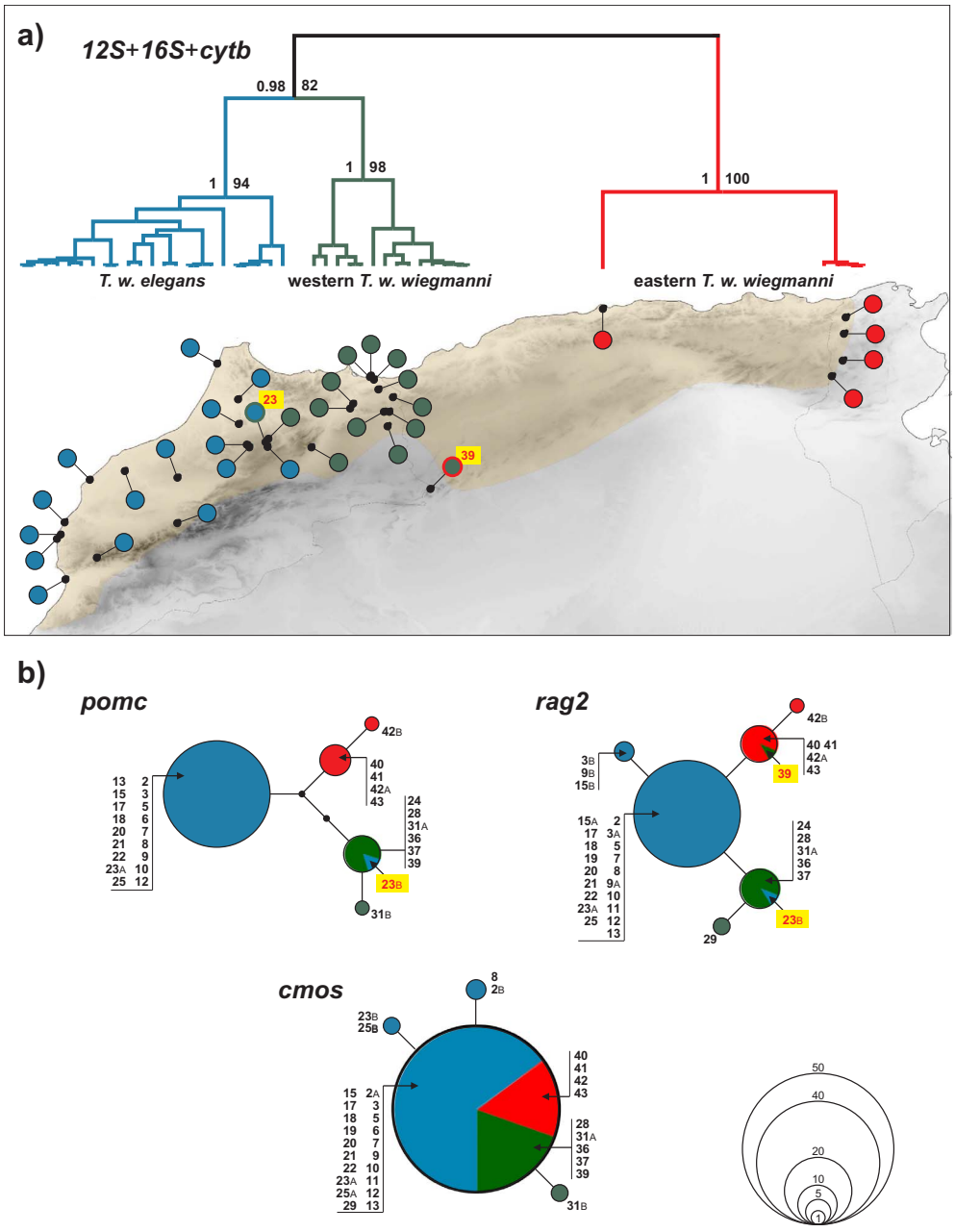

Fig. 2. (a) Bayesian tree depicting the phylogenetic relationships among combined mitochondrial sequences (*12S + 16S + cytb*; 1123 base pairs, bp) of *Trogonophis wiegmanni* (the tree was rooted using *Diplometopon zarudnyi;* GenBank accession numbers: MG661254-MG661259). For the major lineages, Bayesian posterior probabilities (BPP) and bootstrap support values (BS) from Maximum Likelihood analyses are given left and right, respectively, to the corresponding branches. The filled circles in the map show the geographic distribution of the main lineages: the two labelled samples outlined in green (sample 23) and red (sample 39) indicate admixed individuals (see below). (b) Haplotype parsimony networks showing the phylogenetic relationships among haplotypes of the nuclear genes *pomc* (478 bp), *rag2* (798 bp) and *cmos* (350 bp). Haplotypes are represented by pie diagrams with size proportional to their frequency (the haplotype size/frequency ratio is shown in the bottom right inset). For each haplotype, labels indicate sample composition (the letter A or B refer to distinct alleles in heterozygous individuals; for sample codes see Table 1 and Fig. 1) and colours represent mitochondrial lineage assignment of populations (as defined by the Bayesian tree showed above). Red codes included in yellow boxes indicate single instances of nuclear haplotype sharing between populations belonging to distinct mitochondrial lineages and are reported also in the map above. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Phylogenetic analyses

The mitochondrial alignment included 31 haplotypes, while the nuclear gene alignments had between four (*cmos*) and six (*rag2*) haplotypes. GenBank accession numbers are given in Table 1; length and number of variable positions for each gene alignment are provided in Table S2. The phi test did not identify recombination in any of the nuclear gene alignments (in all cases, $p > .05$).

Bayesian and ML phylogenetic analyses recovered three highly supported mitochondrial lineages (BPP = 1; BS ≥ 94) with a strict geographical association (Fig. 2): two lineages within Morocco, including either western samples (1–23 and 25) or eastern samples (24 and 26–39), and a third lineage comprising samples from central Algeria and Tunisia (40–44). The two lineages from Morocco formed a monophyletic clade in both Bayesian and ML trees (BPP = 0.98; BS = 82). With few exceptions (among samples 17–25), all individuals grouped in the western Morocco lineage were identified in the field as *T. w. elegans* (hereafter '*elegans* lineage'), whereas individuals identified as *T. w. wiegmanni* are included in two lineages, one with Tunisian and Algerian samples (hereafter 'eastern *wiegmanni* lineage') and the other one with samples from eastern Morocco (hereafter 'western *wiegmanni* lineage') (Fig. 2). Genetic distance between lineages ranges from 3.8 to 5.3% for *12S* and 8.1–11.2% for *cytb* (Table 2). Each lineage shows high genetic variation with a number of geographically restricted sub-lineages (e.g. the Algerian and the Tunisian sub-lineages, Fig. 2). The split between the eastern *wiegmanni* lineage and the Moroccan lineages is estimated at 4 Ma (High Posterior Density interval, HPD: 2.8–5.5) while the split between the *elegans* and western *wiegmanni* lineages occurred 3 Ma (HPD: 2–3.9). The TMRCAs of the three main lineages are placed in a short time frame between 1.2 and 1.5 Ma (HPD intervals from 0.74 to 3.1). These estimates are congruent among different calibration strategies (see Material and Methods for the calibration approaches used).

Phylogenetic networks based on the nuclear loci *pomc* and *rag2*

show fixed nuclear haplotype differences between individuals belonging to distinct mitochondrial lineages, with two exceptions regarding samples 23 and 39 (marked in Fig. 2). The *cmos* locus shows low phylogenetic resolution, with one common haplotype (90%) widespread across the entire range and three rare (< 5%) derived haplotypes observed in 1–2 samples each.

The multilocus species tree supports the same phylogenetic relationships as the mtDNA tree, with the two Moroccan lineages forming a clade (BPP = 0.9). Divergence time estimates from the species tree analyses largely overlap those based on the mtDNA tree: the root of the tree is placed at 3 Ma (HPD: 2–4.2) and the split between Moroccan lineages at 2.15 Ma (HPD: 1.3–3.1).

3.2. Morphological analyses

A preliminary multivariate analysis (PCA) of the continuous characters showed some differentiation among representatives of the three lineages, though the three groups showed high overlap across the PC1-PC2 bivariate space, which explain 54.6% of the total variation (Fig. 3a). Variables contributing the most to the individual variation across PC1 were head shape and mid-body scales, while across PC2 and PC3 were the remaining scale counts (BS, TA, OS; PCS; Table 4). At the univariate level, specimens of the eastern *wiegmanni* lineage were larger than those of the *elegans* and western *weigmanni* lineages (permANOVA, $F = 5.016$, $p = .011$; Table 3). Specimens of the *elegans* lineage had more robust heads (larger, higher and wider) than specimens from the eastern and western *wiegmanni* lineages (HL: $F = 20.053$, $p = .001$; HW: $F = 15.146$, $p = .001$; HH: $F = 14.282$, $p = .001$), whereas the eastern and western *wiegmanni* lineages did not differ in head shape (pairwise post hoc comparisons: *elegans* vs *wiegmanni*, in all cases $p < .05$; eastern vs western *wiegmanni*, in all cases $p > .05$).

None of the scale counts were correlated with body size (all Spearman tests, $p > .05$). The number of the upper (4) or lower-labial (3) scales was not variable across specimens (Table 3). The number of body scales differed among lineages ($F = 53.869$, $p = .001$; post hoc comparisons between lineages $p < .01$). Eastern and western *wiegmanni* lineages had different, almost non-overlapping, numbers of body scales (eastern: 138–148; western: 147–157), while the *elegans* lineage had an intermediate number of scales (141–152). The number of scales around mid-body ($F = 7.9876$, $p = .001$) and the number of tail annuli ($F = 8.3775$, $p = .002$) were significantly different, although overlapping, among lineages.

Regarding categorical variables, Multiple Correspondence Analysis (MCA) showed that specimens of the eastern *wiegmanni* lineage had a quite distinct scales arrangement relative to most specimens of the western *wiegmanni* and *elegans* lineages (Fig. 3b). These differences among lineages were analysed at the univariate level (Table S3). None of the specimens from the eastern *wiegmanni* lineage had the two postmental scales (CSLS) in contact, while most specimens from the

Table 2
Average genetic distance (*p*-distance) between the main lineages of *Trogonophis wiegmanni* at the three mitochondrial loci: *cytb* (below the diagonal), *16S* and *12S* (above the diagonal; *16S*: first line, *12S*: second line). Standard error estimates are shown in parentheses and were obtained by a bootstrap procedure (1000 replicates).

	elegans lineage	western *wiegmanni* lineage	eastern *wiegmanni* lineage
elegans lineage	–	0.039 (0.007) 0.043 (0.009)	0.051 (0.009) 0.038 (0.008)
western *wiegmanni* lineage	0.081 (0.013)	–	0.050 (0.008) 0.053 (0.010)
eastern *wiegmanni* lineage	0.112 (0.016)	0.100 (0.015)	–

Table 3
Morphological and meristic variation among the three lineages of *Trogonophis wiegmanni* detected in this study (Fig. 2). For each lineage, minimum, maximum and mean values (SD = Standard Deviation) were calculated. For scale position characters, numbers in brackets represent the number of individuals assigned to each category.

Morphological and meristic variables	*Trogonophis elegans*			*Trogonophis wiegmanni*					
				Western (Morocco)			Eastern (Algeria and Tunisia)		
	Mean ± SD	Min–max	n	Mean ± SD	Min–max	n	Mean ± SD	Min–max	n
Linear measurements (in mm)									
Total body length (BL)*	119.89 ± 29.66	87.15–192.05	26	123.72 ± 24.79	78.10–161.58	29	148.57 ± 29.84	79.20–181.20	17
Snout-vent length (SVL)	110.04 ± 27.88	79.5–179.53	26	114.05 ± 23.50	70.79–149.13	29	136.71 ± 27.72	72.8–171.5	17
Head length (HL)	5.76 ± 1.15	4.47–8.42	26	5.18 ± 0.78	3.73–6.66	29	5.95 ± 0.67	4.40–7.00	17
Head height (HH)	4.69 ± 1.19	3.5–7.56	26	4.29 ± 0.75	3.09–5.79	29	4.70 ± 0.85	3.0–5.88	17
Head width (HW)	5.52 ± 1.48	4.29–9.34	26	4.89 ± 0.90	3.36–6.71	29	5.56 ± 0.80	4.00–6.87	17
Scale counts									
Body (BS)	147.54 ± 2.47	141–152	26	151.48 ± 2.41	147–157	29	142.27 ± 3.20	138–148	11
Mid-body (MBS)	56.46 ± 3.28	49–66	26	53.21 ± 2.44	49–59	28	53.40 ± 4.98	48–60	5
Tail (TA)	12.28 ± 0.84	11–14	25	12.32 ± 0.72	11–13	28	11.27 ± 0.79	10–12	11
Upper labials (ULS)	–	4		–	4	29	–	4	11
Lower labials (LLS)	–	3		–	3	28	–	3	11
Ocular scales (OS)	5.19 ± 0.57	4–7	26	5.38 ± 0.78	5–8	29	4.82 ± 0.75	4–6	11
Post-chin shield (PCS)	5.08 ± 0.39	4–6	26	4.93 ± 0.53	4–6	29	4.82 ± 0.40	4–5	11
Scale position									
Contact between postmental scales (CSLS)	No (2)/single-point (6)/yes (9)			No (0)/single-point (14)/yes (15)			No (10)/single-point (1)/yes (0)		
Extra scale between postmental scales (EXSLS)	No (17)/yes (1)			No (29)/yes (0)			No (8)/yes (3)		
Extra scale between pre and post frontal scales (EXBPPF)	No (26)/yes (0)			No (29)/yes (0)			No (10)/yes (1)		
Extra scale between postfrontal and body scales (EXPFB)	No (12)/yes (14)			No (27)/yes (2)			No (9)/yes (2)		

Linear measurements: BL, total body length from the tip of the snout to the tip of the tail; SVL, snout-vent length from the tip of the snout to the cloaca opening; HH, head height at its highest point; HW, head width at its widest point; HL, head length from the tip of the rostral scale to the end of the post-frontal scales (*: BL is included in the table only for descriptive purposes, but was not included in the morphological analyses due to its correlation to SVL).
Meristic characters: BS, number of scales along the body from the post-frontal to the cloacal scales excluded; MBS, number of scales around the mid-body counted from a mid-body central annuli; TA, number of annuli in the tail from the cloaca to the tip of the tail; ULS, number of upper labial scales; LLS, number of lower labial scales; OS, number of ocular scales; PCS, number of post-chin shield scales; CSLS, contact between the two postmental scales (yes = in broad contact, single-point = in a single point of contact, no = not in contact); EXSLS, presence of an additional scale between postmental scales (yes = presence, no = absence); EXBPPF, presence of an additional scale in the middle of the frontal scales (yes = presence, no = absence); EXPFB, presence of an additional scale between postfrontal and body annuli scales (yes = presence, no = absence).

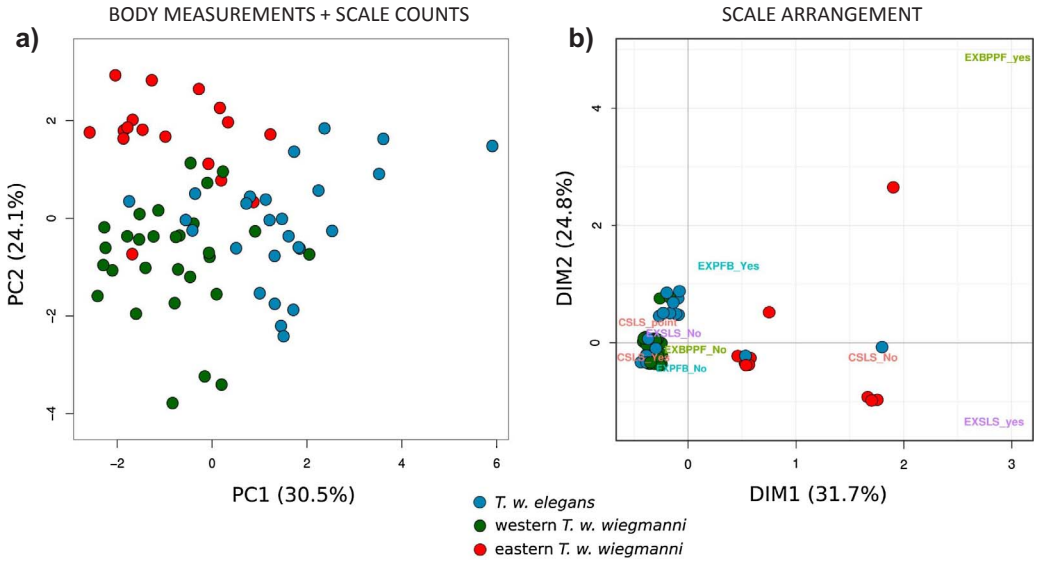

Fig. 3. Results of the multivariate analyses of the continuous and categorical variables (a) Principal Components Analysis (PCA) plot between PC1 and PC2 based on linear body measurements and scale counts. (b) Multiple Correspondence Analysis (MCA) plot (first two dimensions) based on the scale arrangement. Dots represent individuals and are coloured according to Fig. 2. For abbreviations of the categorical variables see Table 3.

Table 4
Principal Components Analysis (PCA) results: correlations between the first three principal components (PC1, PC2 and PC3) and the linear body measurements and scale counts. Values in bold represent the most contributing variables. For each axis, eigenvalues, variance (% variance) and cumulative variance (% cum variance) are provided. For abbreviations of linear measurement and meristic variables see Table 3.

	PC1	PC2	PC3
SVL	−0.049	0.346	−0.324
HL[a]	**0.484**	0.196	−0.040
HW[a]	**0.545**	0.091	0.149
HH[a]	**0.518**	0.078	0.281
BS	−0.021	**−0.496**	0.289
MBS	**0.402**	−0.185	−0.217
TA	0.068	**−0.555**	0.131
OS	0.080	**−0.447**	−0.243
PCS	0.158	−0.199	**−0.765**
Eigenvalues	2.747	2.166	1.019
% variance	30.52	24.07	11.33
% cum variance	30.52	54.59	65.91

[a] Residuals.

other two lineages did. Specimens of the *elegans* lineage showed an extra scale behind the post-frontals (EXPFB) more frequently than the other lineages. Finally, the presence of additional scales between the postmental or frontal scales was not associated to any lineage.

4. Discussion

4.1. Cryptic species within Trogonophis wiegmanni

The phylogenetic and morphological patterns observed in the checkerboard worm lizard *Trogonophis wiegmanni* are consistent with the expectation of strong phylogeographic structure and cryptic diversity in fossorial reptiles (e.g. Albert and Fernández, 2009) as well as with the prediction of undescribed reptile diversity within the Maghreb (Ficetola et al., 2013).

We found three main phylogenetic lineages which are reciprocally monophyletic at mitochondrial loci and also sorted at fast-evolving nuclear loci: the *elegans* lineage in central and western Morocco (samples 1–22, 25), the western *wiegmanni* lineage in eastern Morocco (samples 24, 26–38) and the eastern *wiegmanni* lineage in Algeria and Tunisia (samples 40–44). The former two lineages co-occur in a narrow zone in central Morocco (including samples 17–25) where a single instance of mito-nuclear mismatch is observed in sample 23, which carries mitochondrial haplotypes diagnostic of the *elegans* lineage, but is heterozygous for diagnostic nuclear alleles of the *elegans* and the western *wiegmanni* lineages (Fig. 2) and showed an intermediate phenotype between the two subspecies (Table 1). Moreover, while outside of this narrow contact zone Moroccan lineages are congruent with the typical colouration of either subspecies ('mauve' for samples 1–16 of the *elegans* lineage and 'yellow' for samples 26–38 of the western *wiegmanni* linage), some individuals from this region (samples 18, 19, 23 and 25) show yellow hues but genetic traits diagnostic of the *elegans* lineage. The occurrence of individuals with an intermediate phenotype and mixed genotypes between two lineages near their putative contact zone is suggestive of introgression following hybridization. A similar situation probably applies to sample 39, which is located in the area of contiguity between the western and eastern *wiegmanni* lineages and shows nuclear haplotypes of both lineages and mitochondrial haplotypes of the western *wiegmanni* lineage.

Mitochondrial genetic distance between *Trogonophis* lineages (Table 2) is similar to mitochondrial divergence observed between other amphisbaenian species (e.g. Albert and Fernández, 2009; Sindaco et al., 2014; Teixeira et al., 2014). The fact that also nuclear genealogies demonstrate lineage sorting is further evidence that these lineages have a long history of independent evolution and reproductive isolation. According to the Unified Species Concept (de Queiroz, 2007) the three evolutionary lineages within *T. wiegmanni* may represent distinct species. Properties such as intrinsic reproductive isolation, diagnosability and monophyly serve as important lines of evidence relevant to assess the separation of lineages and therefore to species delimitation. While intensive sampling in Algeria and a comprehensive morphological assessment will be needed for a correct delimitation and formal species description, preliminary morphometric and meristic data show some significant differences among them (Tables 3 and 4; Supplementary Table S3; Fig. 3). Lack of prominent morphological differences between fossorial sister taxa is an expected pattern due to their oversimplified morphology resulting from the adaptation to a burrowing lifestyle (see e.g. Gans, 1977).

Diversification within *Trogonophis* took place in the Pliocene with an older split between the western and eastern Maghreb lineages (3–4 Ma) relative to the diversification within the western Maghreb (2–3 Ma). North Africa was affected by rapid alternations of humid and hyperarid phases during the mid-Pliocene to the Pleistocene (Street and Gasse, 1981; Kowalski, 1991; Quezel and Barbero, 1993; deMenocal, 2004; Schuster et al., 2006), which may have played a central role in the isolation and gene flow disruption between ancestral *Trogonophis* populations. Considering the bioclimatic requirements of this species, which encompasses Mediterranean and arid regimes but is precluded by

hyper-arid or Saharan conditions (Sánchez and Escoriza, 2014), hyper-arid phases would have compromised the continuity of habitat and climatic conditions suitable for this species and promoted allopatric divergence between populations from separate refugia. A recent study indicates that the subspecies *T. w. elegans* and *T. w. wiegmanni* are not segregated in terms of bioclimatic niche (Sánchez and Escoriza, 2014), and therefore we may expect that stages of extreme aridification induced contractions of ancestral *Trogonophis* populations throughout the entire range. Within Morocco, prominent topographic barriers such as the Atlas and the Rif mountains have probably limited the subsequent expansion of lineages during the mesic periods of the Plio-Pleistocene. Indeed, Moroccan lineages have established a secondary contact in the plateau located between the northern slope of the Middle Atlas and the southern slope of the Rif (Fig. 2), whereas south of the Atlas Mountains climatic conditions have not been suitable for their survival or (re)colonisation.

4.2. Biogeographic and biodiversity patterns within the Maghreb

To date, we have still a blurred picture of biodiversity patterns within the Maghreb and a poor understanding of the main processes responsible for their formation. While in recent decades intense phylogeographic research has clarified the main role played by Plio-Pleistocene climatic oscillation and orographic barriers in shaping the structure of the biodiversity in the European portion of the Western Palaearctic, comparatively little is known about the processes underlying the phylogeographic patterns of the fauna and flora of North Africa (Hewitt, 2000; Husemann et al., 2014). Emerging evidence from this study and available literature on taxa widespread across the Maghreb support (i) a major biogeographic partition between western and eastern Maghreb and (ii) a role of the Atlas as a biogeographic divide within the western Maghreb (Morocco). The latter is a well-established pattern by many phylogeographic studies with a dense sampling in Morocco (Brown et al., 2002; Fritz et al., 2006; Fonseca et al., 2009; Habel et al., 2012; Perera et al., 2012; Sousa et al., 2012; Pedroso et al., 2013; Husemann et al., 2014; Lansari et al., 2015; Veríssimo et al., 2016; Rosado et al., 2017). The existence of a biogeographic divide between western and eastern Maghreb was initially proposed by cross-taxa studies focused on terrestrial (de Jong, 1998) and freshwater (Doadrio, 1994) fauna. Since then, a deep split between western (Morocco) and eastern (W Algeria and Tunisia) populations has been documented across different organisms within the Maghreb, although in some cases, such as this study, the lack of dense sampling in Algeria precludes delimiting the exact ranges of these genetic lineages. Previously, when very few phylogeographic studies were available for the Maghreb, some authors argued that such a bipartite east-west differentiation could be too simplistic, reflecting incomplete locality sampling rather than real geographic differentiation (Fritz et al., 2009). However, although the lack of dense sampling in Algeria still persists in phylogeographic studies, we can now list many examples among the Maghreb fauna in which the main western and eastern lineages are found in close proximity rather than separated by a large gap in geographical sampling. These examples provide strong support for the observed west-east pattern and fairly good geographical resolution for the limits between the western and the eastern genetic lineages.

Among taxa strictly endemic to the Maghreb, in the lacertid lizards *Timon pater/T. tangitanus* (Paulo et al., 2008; Perera and Harris, 2010b) and *Psammodromus microdactylus/P. blanci* (Mendes et al., 2017), the gecko *Ptyodactylus oudrii* (Perera and Harris, 2010; Metallinou et al., 2015), the salamander *Salamandra algira* (Ben Hassine et al., 2016) and the frogs *Discoglossus scovazzi/D. pictus* (Vences et al., 2014a) the main east-west phylogeographic break is found in eastern Morocco, commonly along the arid valley of the Moulouya River. In these species populations from easternmost Morocco belong to the same or to a closely related lineage of those from eastern Algeria and Tunisia. In other endemics such as *Trogonophis* (this study) and the butterfly *Melanargia lucasi* (Habel et al., 2017), the main phylogeographic break could be either in western or in central Algeria, such as in the case of the North African water frog *Pelophylax saharicus* that shows a transition between the main lineages in proximity of the Kabylian region (central-eastern Algeria) (Nicolas et al., 2015). Also for the Kabylian area, phylogeographical (lineage) breaks have been documented across multiple taxa (e.g. Carranza and Wade, 2004; Barata et al., 2008; Fritz et al., 2009; Guiller and Madec, 2010; Nicolas et al., 2014a). In all of these species, it is unlikely that additional sampling in Algeria would reject the observed bipartite east-west differentiation pattern.

This pattern is observed also in other taxa with a North African primary range but distributed also outside the Maghreb. These include the snakes *Natrix maura* and *Hemorrois hippocrepis* (Barata et al., 2008; Carranza et al., 2006), the skink *Chalcides ocellatus* (Kornilios et al., 2010), the frog *Hyla meridionalis* (Recuero et al., 2007), the shrews *Crocidura russula/C. pachyura* (Cosson et al., 2005; Nicolas et al., 2014a) and the lark *Chersophilus duponti* (García et al., 2008). In contrast, in the terrapin *Mauremys leprosa* (Veríssimo et al., 2016), in geckos of the *Tarentola mauritanica* complex (Rato et al., 2016) and in *Daboia* vipers (Martínez-Freiría et al., 2017), all Maghrebian lineages are found in Morocco indicating a centre of diversification in the western Maghreb rather than a pattern of east-west differentiation.

Finally, concerning species with a secondary range in North Africa, taxa with an European origin such as *Coronella girondica* (Santos et al., 2012), *Emys orbicularis* (Stuckas et al., 2014) and *Bufo spinosus* (García-Porta et al., 2012; Recuero et al., 2012) show distinct lineages in western and eastern Maghreb; others such as the viper *Vipera latastei* (Velo-Antón et al., 2012) and the tortoise *Testudo graeca* (Fritz et al., 2009), with an European and Caucasian origin respectively, show three or four lineages from western to eastern Maghreb with a similar degree of differentiation among them.

Therefore, the comparative analysis of available studies on North African species belonging to different biogeographic categories (endemics or native to the Maghreb, or with an origin outside the Maghreb) points to an east-west phylogeographic paradigm, with Moroccan populations being well-differentiated from their eastern Algerian and Tunisian counterparts (see also comparisons by Barata et al., 2008; Guiller and Madec, 2010; Nicolas et al., 2015). Such concordance has been often used as a criterion for identifying common evolutionary and biogeographic phenomena (Avise, 2000; Arbogast and Kenagy, 2001; Hewitt, 2004). However, shared distributions of lineages across taxa does not necessarily mean that they have been originated as a consequence of the same biogeographical events (see e.g. Papadopoulou and Knowles, 2016; Salvi et al., 2016). Indeed, both the Moulouya and the Kabylian regions suffered dramatic geographic and environmental changes which took place at different times. Since the Late Miocene the formation of fossil islands and marine transgression (in correspondence of the Rif block, west to the Moulouya region, and the Edough Peninsula, east to the Kabylian region) may have provided opportunities for lineage isolation and divergence (see Arano et al., 1998; Carranza and Wade, 2004; Veith et al., 2004; Paulo et al., 2008). During Pleistocene climatic oscillations, the sharp environmental transitions that occurred in correspondence with the Moulouya River Basin and the Kabylian area may have established more recent divergence or prevented the admixture between previously differentiated lineages (e.g. Fritz et al., 2009; Nicolas et al., 2015; Vences et al., 2014a). In fact, both Miocene (e.g. in lizards and salamanders: Carranza and Wade, 2004; Paulo et al., 2008; Ben Hassine et al., 2016; Mendes et al., 2017) and Plio-Pleistocene (e.g. in frogs, tortoises, mammals and birds: Cosson et al., 2005; Garcia et al., 2008; Fritz et al., 2009; Nicolas et al., 2015), divergences have been inferred between western and eastern lineages in distinct taxa. Moreover, while in some species these areas may have acted as barriers triggering the primary vicariant divergence between western and eastern lineages (e.g. Barata et al., 2008; Cosson et al., 2005; Paulo et al., 2008; Nicolas et al., 2015), in other species they likely acted as a secondary barrier for the expansions of

lineages that diversified elsewhere (e.g. Guiller and Madec, 2010; Nicolas et al., 2014b; Vences et al., 2014a).

In conclusion, while some common patterns emerged across Maghrebian taxa, it is still tentative to make a link between such patterns and common biogeographic and evolutionary processes. Major drawbacks in this respect include the limited number of studies with a dense sampling across the Maghreb, and the scarcity or absence of fossil records that prevents the inference of historical distribution patterns of Maghrebian taxa and reliable estimates of divergence time between main lineages. This latter aspect is particularly evident when the estimates of the divergence time between lineages encompass large confidence intervals (e.g. Paulo et al., 2008; Nicolas et al., 2015) or large differences between studies as for example in the cases of *Salamandra algira* (Beukema et al., 2010; Vences et al., 2014b; Ben Hassine et al., 2016), *Pleurodeles* spp. (Carranza and Wade, 2004; Veith et al., 2004; Escoriza et al., 2016), *Natrix maura* (Guicking et al., 2006; Fritz et al., 2012) and *Agama impalearis* (Brown et al., 2002; Gonçalves et al., 2012). Therefore, additional studies across multiple taxa are needed to delimit these main biogeographic units within the Maghreb and their association with past climate, topography and geology. In the specific case of *Trogonophis wiegmanni* future studies should focus on covering the sampling gap in western Algeria and gathering more morphological data for a comprehensive assessment of the taxonomic status and distribution of the three main lineages.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.11.013.

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